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ISOLATION BY IMS PROCEDURE AND MOLECULAR CHARACTERISATION OF ESCHERICHIA COLIOI57:B7

ROZILA BINTI ALIAS

ISOLATION BY IMS PROCEDURE AND MOLECULAR CHARACTERISATION OF *ESCHERICHIA COLI* O157:H7

By

ROZILA BINTI ALIAS

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ROZILA BINTI ALIAS

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Chairman : Son Radu, Ph.D.

Faculty : Food Science and Biotechnology

Escherichia coli O157:H7 was discovered in association with the outbreak of hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura in 1982. Since then, this organism has become a very important cause of foodborne infection in the developed countries. The Immunomagnetic Bead Separation (IMS) and QUIX™ strip test O157 techniques were used to detect *E. coli* O157:H7 in the 40 samples of frozen beef. Twenty nine out of 40 samples of frozen beef (72.5%) were presumptive positive with the strip QUIX O157 strip. 18 of the 29 presumptive positive samples were confirmed positive by using immunomagnetic bead separation (IMS) and conventional

methods. A total of 123 isolates from the 18 positive samples were further characterised using plasmid, antibiotic profiling and randomly amplified polymorphic DNA (RAPD). All the strains (100%) were resistant to bacitracin, cephalothin and penicillin. Others, 85.4, 81.3, 70.0, 47.2, 41.5, 32.5, 27.6, 23.6 and 12.2% were resistant to carbenicillin, erythromycin, streptomycin, kanamycin, nalidixic acid, tetracycline, chloramphenicol, ampicillin and gentamycin, respectively. The multiple antibiotic resistance index (MAR) for *E. coli* O157:H7 isolates ranged between 0.38 to 0.92. 123 isolates were grouped into sixty six resistotypes with resistance to five or more antibiotics. 97.3% of *E. coli* O157:H7 strains contained plasmid DNA ranging in size from 1.34 MDa to 60 MDa. Based on the plasmid size, the strains could be grouped into 5 groups or profiles; profile I contained 60 MDa and 2.5 MDa (49.6%); profile II, 60 MDa (43.1%), profile III and IV, 60 MDa and small plasmids and profile O represented plasmidless strains. A 60 MDa plasmid which has been identified as a serotype specific for *E. coli* O157:H7 was detected in all the 123 strains. Ten primers were screened but only three primers, Gen1-50-02, Gen1-50-09 and Gen1-50-10 were chosen for PCR reactions because they gave reproducibility PCR products with one, five and six RAPD patterns respectively. These amplified fragments ranged from 0.25 to 4.0 kb in sizes.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains

PEMENCILAN SECARA PROSEDUR IMS DAN PENCIRIAN MOLEKULAR *ESCHERICHIA COLI* O157:H7

Oleh

ROZILA BINTI ALIAS

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Pengerusi : Son Radu, Ph.D.

Fakulti : Sains Makanan dan Bioteknologi

Escherichia coli O157:H7 telah dikenalpasti terlibat dalam implikasi dengan hemorrhagic colitis, hemolytic uremic syndrom dan thrombotic thrombocytopenic purpura semenjak dari tahun 1982. Sejak itu, organisma ini telah menjadi begitu penting dalam keracunan makanan dikebanyakkan negara membangun. Teknik pemisahan manik immunomagnetik (IMS) dan ujian jalur QUIX™ O157 telah digunakan bagi mengesan *E. coli* O157:H7 di dalam 40 sampel daging beku. Duapuluh sembilan daripada 40 sampel daging beku (72.5%) adalah positif dengan ujian jalur O157. 18 daripada 29 sample yang positif telah dikenalpasti positif secara pemisahan manik immunomagnatik (IMS) dan kaedah tradisional. Sejumlah 123 isolat daripada 18 sampel positif telah diteruskan penciriannya menggunakan plasmid, antibiotik profil dan pempolimorfik DNA secara rawak (RAPD). Kesemua (100%) strain adalah rintang terhadap antibiotik

bacitracin, cefalotin dan penisilin. Selebihnya, 85.4, 81.3, 70.0, 47.2, 41.5, 32.5, 27.6, 23.6 dan 12.2% rintang terhadap karbenisilin, eritromisin, streptomisin, kanamisin, asid nalidisik, tetrasiklin, kloramfenikol, ampisilin dan gentamisin. Nilai MAR bagi *E. coli* O157:H7 yang dipencilkan adalah diantara 0.38 hingga 0.92. 123 isolat dibahagikan kepada enampuluh enam resistotype dengan kerintangan terhadap lima atau lebih antibiotik. 97.3% daripada strain *E. coli* O157:H7 mengandungi jalur DNA plasmid yang bersaiz diantara 1.34 MDa hingga 60 MDa. Berdasarkan saiz plasmid ini, isolat dapat dikategorikan kepada lima jenis kumpulan atau profil, profil I mengandungi 60 MDa dan 2.5 MDa (49.6%); profil II, 60 MDa (43.1%), profil III dan profil IV, 60 MDa dan beberapa plasmid kecil dan profil O menunjukkan strain yang ketiadaan plasmid. Plasmid 60 MDa telah ditentukan sebagai spesifik serotype bagi *E. coli* O157:H7. Sepuluh jenis primer telah diujijaya tetapi hanya tiga primer yang terpilih dalam tindakbalas PCR kerana ia memberikan reproduksi PCR yang tinggi dimana satu, lima dan enam jenis paten RAPD diperlihatkan. Fragmen yang telah diamplifikasikan bersaiz dari 0.25 hingga 4.0 kb.

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I certify that an Examination Committee met on 23 October, 1999 to conduct the final examination of Rozila Bt Alias on her Master Thesis entitled "Isolation by IMS Procedure and Molecular Characterisation of *Escherichia coli* O157:H7" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

HIRZUN MOHD YUSOF, Ph.D.
Faculty of Food Science and Biotechnology
University Putra Malaysia
(Chairman)

SON RADU, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

GULAM RUSUL RAHMAT ALI, Ph.D.
Professor / Dean
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

ZAITON HASSAN, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

MOHD. GHAZALI MOHAYIDIN, Ph.D.
Professor/Deputy Dean of Graduate School

Date: 3 MAR 2000

This thesis was submitted to the Senate of Universiti Putra Malaysia and was accepted as fulfilment of the requirements for the degree of Master of Science.

KAMIS AWANG, Ph.D.
Associate Professor,
Dean of Graduate School,
Universiti Putra Malaysia.

Date: 11 MAY 2000

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

(ROZILA BT ALIAS)

Date: 22/2/2000

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CHAPTER I

INTRODUCTION

Verocytotoxin-producing *Escherichia coli* (VTEC) O157:H7 is the major cause of human infection. Lately, *E. coli* O157:H7 has been recognized as agent of haemorrhagic colitis (HC), which can progress to the hemolytic-uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) (Griffin and Tauxe, 1991).

Outbreaks caused by *E. coli* O157:H7 may fall into two categories which involves person-to-person spread and foodborne. Most outbreaks have been reported to be associated with foods of bovine origin, particularly raw meat (Wells *et al.*, 1983; Xu *et al*, 1990; Belongia *et al.*, 1991; Bolton *et al.*, 1996) because of contamination and improperly cooked beef. This organism has also been recovered from unpasteurized milk, faeces, contaminated vegetables, water, apple cider and other more novel foods which have become contaminated (Karmali, 1989; Griffin and Tauxe, 1991; Swerdlow *et al.*, 1992; Besser *et al.*, 1993; Feng, 1995).

Since isolation of *E. coli* O157:H7 have been important, a rapid method for detection and laboratory diagnosis of *E. coli* O157:H7 whether in clinical, food or environmental samples has been developed over recent years with the use of liquid

enrichment and the development of methods such as immunomagnetic beads separation and strip test O157 (Okrend *et al.*, 1992; Matlock, 1994; Wallace *et al.*, 1997). Identification of the source of contamination of foods with *E. coli* O157:H7 is important to the understanding of the epidemiology of human infection and devising strategies for its control.

For epidemiolgical investigations, highly discriminatory typing methods are required to provide useful information regarding common cases and the identification of sources of infection. Conventional typing methods are not very discriminatory because they are dependent on expressed and possible variable phenotic features. Nowadays, PCR-based DNA fingerprinting methods such as randomly amplified polymorphic DNA (RAPD) analysis were introduced and was reported to be one of the most reliable methods in distinguishing between bacterial species and strains.

Objectives

Thus, this research was undertaken to determine the prevalence of *E. coli* O157:H7 in imported frozen beef using strip test QUIX O157 and immunomagnetic bead separation methods. The *E. coli* O157:H7 strains isolated were characterized by their plasmid profiles, antibiotic resistance and randomly amplified polymorphic DNA (RAPD) patterns.

CHAPTER II

LITERATURE REVIEW

Escherichia coli O157:H7

E. coli O157:H7 has a rigid structure to maintains the rod-like shape of about 2.0 μm in length and 1.0 μm in diameter (Glass, 1982). Genome size (including chromosomal and extrachromosomal DNA) in *E. coli* strain varies from 2.3 x 10^9 to 3.0 x 10^9 daltons. The G + C content ranges from 49% to 52% (Selander *et al.*, 1987). Another characteristics of *E. coli* O157:H7 is that it carries a number of common pili (or fimbriae). These appear to be responsible for tissue 'stickiness' and thus for pathogenicity of virulent species. The sex pili participate in the transfer of genetic material between bacteria (conjugation). Their synthesis is genetically determined by extra-chromosomal DNA rather than by bacteria genetic apparatus itself. Sex pili have doubtful distinction of acting as receptors for certain bacteria phages.

E. coli O157:H7 have two surface components which form the basis for the serological classification system: O antigen of lipopolysaccharide (LPS) and H of flagella (H). The O antigen identifies the serogroup of a strain and the H antigen identify its serotype.

Currently, there are five virotypes causes by *E. coli*; enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and enterohaemorrhagic *E. coli* (EHEC) or verotoxigenic *E. coli* (VTEC).

Enterohaemorrhagic *E. coli* (EHEC) or Verotoxigenic *E. coli* (VTEC)

A major recent development in the field of enteric infections due to *E. coli* has been the recognition of a new group of pathogenic *E. coli* that produce verotoxin or Shiga-like toxin. This strain which produce toxin harmful to cultured Vero cells (African green monkey kidney cells) are called Verotoxin-producing *E. coli*, or VTEC, and these can cause haemorrhagic colitis in humans which usually presents as bloody diarrhoea. In the EHEC group of strains, there is one predominant serogroup and serotype, *E. coli* O157:H7.

In 1982, two outbreak of bloody diarrhoea in Oregon and Michigan (Riley *et al.*, 1983) and another outbreak in Ottawa, Canada (Steward *et al.*, 1983) led to the recognition of a new pathogenic serotype, *E. coli* O157:H7. Johnson *et al.* (1983) were the first to report that *E. coli* O157:H7 produce verotoxins (VT). Studies have revealed the presence of at least two toxins, VTI and VTII which because of their similarity to Shiga toxin have also been called Shiga-like toxins, SLTI and SLTII.

Escherichia coli O157:H7 Diseases

Hemorrhagic colitis (HC)

Hemorrhagic colitis is typically a self-limiting, acute bloody diarrhoea that begins with stomach cramps and watery diarrhoea after an incubation period of 3-8 days. It can be distinguished from inflammatory colitis by the lack of fever and absence of leukocytes in the stools. It affects mainly adults, with a peak incidence in the summer months, and can be life-threatening in the elderly (Ryan *et al.*, 1986; Carter *et al.*, 1987; Morgan *et al.*, 1988; MacDonald *et al.*, 1996).

Haemolytic Uremic Syndrome (HUS)

Hemolytic uremic syndrome is characterized by three features, acute renal failure, haemolytic anaemia (reduction in the number of red blood cells) and thrombocytopaenia (a drop in the number of blood platelets), sometimes preceded by a bloody diarrhoea. It is most common in children among whom it is the leading cause of acute renal failure in Western Europe and North America. In 70 cases seen in London between 1980 and 1986 the fatality rate was 6%, with 13% of cases showing some long term kidney-damage.

Thrombotic Thrombocytopaenic purpura (TTP)

Thrombotic thrombocytopaenic purpura is related to the hemolytic uremic syndrome but includes fever and neurological symptoms.

Methods for Detection of *E. coli* O157:H7

The first selective and differential medium was devised by MacConkey in 1905. However, it has been variously modified. Eosin methylene blue agar is a popular selective medium as indicator for lactose fermentation by forming a precipiatte at low pH. Strong lactose fermenters produce green-black colonies with a metallic sheen. The development of detection methods for *E. coli* O157:H7 which are able to detect at very low levels of *E. coli* O157:H7 have been reported. Selective agars, based on the inability of *E. coli* O157 to ferment either sorbitol (March and Ratnam, 1986) or rhamnose (Chapman *et al.*, 1991) and the absence of beta-glucuronidase activity (Thompson *et al.*, 1990), have been used to isolate *E. coli* O157 from a variety of samples by direct culture. Selectivity of the medium has been improved by the supplementation of Sorbitol MacConkey agar with cefixime in the presence of rhamnose (CR-SMAC) (Chapman *et al.*, 1991) and by the inclusion of potassium tellurite (CT-SMAC) (Zadik *et al.*, 1993). Other differential agar such as SD-39 (QA Life Sciences, Inc.) and Chomagar® O157 (Chomagar) confirmed of *E. coli* O157 by colour.

Several new techniques have been developed such as immunomagnetic separation (IMS) technique using beads coated with polyclonal *E. coli* O157 antibodies (Okrend *et al.*, 1992; Chapman *et al.*, 1994; Matlock, 1994) are commercially available (Dynal). Immunoblotting has also been employed to detect *E. coli* O157 (Willshaw *et al.*, 1993) in which colonies are transferred to nitrocellulose

Table 1: Selective Media for Isolation of *E. coli* O157:H7

Media	Reference
Solid	
SMAC	Farmer and Davis, 1985
HC	Szabo *et al.*, 1986
PRS-MUG	Okrend *et al*, 1990
MSA-BC1G	Okrend *et al.*, 1990
CR-SMAC	Chapman *et al.*, 1991
MSA -MUG	Padhyle and Doyle, 1991
CT-SMAC	Zadik *et al.*, 1993
Liquid	
mTSB	Doyle and Schoeni, 1987
dm TSB-CA	Padhyle and Doyle, 1991
BPW-VCC	Chapman *et al.*, 1993
mEC + n	Organon Teknika, 1993.

Reported from Microbiological Safety of Food, 1995

membranes. An alkaline phosphatase-conjugated O157 antiserum is used for the detection of positive colonies. ELISA method (3M Corporation) is another method which is a rapid sandwich enzyme-linked immunosorbent assay (ELISA) has been described for the detection of O157 VTEC in foods (Padhye and Doyle, 1991). In this test polyclonal O157 antibody is used as the capture antibody and a monoclonal antibody, claimed to be specific for VTEC belonging to serogroups O157 and O26, is used as the detection antibody. Both serogroups can be detected directly from faecal specimens in less than 2 hours by direct immunofluorescence antibody staining (Park *et al*, 1994). Other methods developed to improve the detectabiliy of *E. coli* O157 in food and environmental samples include using of hydrophobic grid membranes filters (HGMF) to permit the screening of a large number of isolates (Doyle and Schoeni, 1987; Todd *et al*., 1988).

Plasmid analysis of *E. coli* O157:H7 also can be used to identify strains in outbreaks and sporadic cases of infection (Scotland *et al*., 1987; Frost *et al*., 1989). However, this method is limited because virtually all O157 VTEC isolates carry a plasmid with a molecular weight of 60 MDa , but some strains may carry additional plasmids. Pulsed-field gel electrophoresis (PFGE) of genomic DNA has been performed on *E. coli* O157:H7 strains from different origins (BÖhm and Karch, 1992; Harsono *et al*., 1993; Krause *et al*., 1996). In general, among the *E. coli* O157:H7 strains the restriction patterns were either identical or differed only by a few fragment bands. The enzymes found to be the most useful in these studies were *Xba*I and *Sfi*I. It was concluded that PFGE should be used together with other typing methods in epidemiological studies of O157 VTEC infections.

The polymerase chain reaction (PCR) is widely used for the detection of enterotoxin genes (Olive, 1989; Victor *et al.*, 1991) and verocytotoxin (VT) genes (Karch and Meyer, 1989; Johnson *et al.*, 1990; Pollard *et al.*, 1990; Woodward *et al.*, 1992). A multiplex PCR assay is one of the type of PCR has frequently been used for the analysis of the shiga-like toxin I (SLT-I) and SLT-II genes in *E. coli* O157:H7 (Meng *et al.*, 1997; Tsen and Jian, 1998). Non-radioactively labelled probes for VT genes also have been developed and enable DNA hybridization to be applied in a wide range of laboratories. The potential exists to use specific PCR to detect toxigenic *E. coli in situ* even in parafin-wax-embedded (Nuovo *et al.*, 1991). Furthermore, primer design influences the range of toxin variants detected and their differentiation (Karch and Meyer, 1989; Johnson *et al.*, 1990, Woodward *et al.*, 1992). Detailed discussions of the potential of PCR technology are presented by Innis and Gelfand (1990) and Birkenmeier and Mushahwar (1991).

Epidemiolgy and Transmission

Initially, *E. coli* O157:H7 has been reported as a cause of sporadic cases and outbreak of diarrhea throughout North America, South America, Western Europe and Asian. Nowadays it has been reported that the infections of this miroorganism have also occurred throughout much of the world (Doyle, 1991). This included Washington State (Hancock *et al.*, 1994), United Kingdom (Frost *et al.*, 1989), Mexico (Cravioto *et al.*, 1990), Argentina (Lopez *et al.*, 1989), Belgium (Piererd *et al.*, 1990), China (Xu *et al.*, 1990), Thailand (Echeverria *et al.*, 1989) and Malaysia (Son *et al.*, 1998).